

Developmental Regulation of Membrane Traffic Organization during Synaptogenesis in Mouse Diaphragm Muscle

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Abstract. In innervated adult skeletal muscles, the Golgi apparatus (GA) displays a set of remarkable features in comparison with embryonic myotubes. We have previously shown by immunocytochemical techniques, that in adult innervated fibers, the GA is no longer associated with all the nuclei, but appears to be concentrated mostly in the subneural domain under the nerve endings in chick (Jasmin, B. J., J. Cartaud, M. Bornens, and J.-P. Changeux. 1989. *Proc. Natl. Acad. Sci. USA.* 86:7218–7222) and rat (Jasmin, B. J., C. Antony, J.-P. Changeux, and J. Cartaud. 1995. *Eur. J. Neurosci.* 7:470–479). In addition to such compartmentalization, biochemical modifications take place that suggest a functional specialization of the subsynaptic GA. Here, we focused on the developmental regulation of the membrane traffic organization during the early steps of synaptogenesis in mouse diaphragm muscle. We investigated by immunofluorescence microscopy on cryosections, the distribution of selected subcompartments of the exocytic pathway, and also of a representative endocytic subcompartment with respect to the junctional or extrajunctional domains of developing myofibers. We show that throughout development the

RER, the intermediate compartment, and the prelysosomal compartment (mannose 6-phosphate receptor-rich compartment) are homogeneously distributed along the fibers, irrespective of the subneural or extrajunctional domains. In contrast, at embryonic day E17, thus 2–3 d after the onset of innervation, most GA markers become restricted to the subneural domain. Interestingly, some Golgi markers (e.g., α -mannosidase II, TGN 38, present in the embryonic myotubes) are no longer detected in the innervated fiber even in the subsynaptic GA. These data show that in innervated muscle fibers, the distal part of the biosynthetic pathway, i.e., the GA, is remodeled selectively shortly after the onset of innervation. As a consequence, in the innervated fiber, the GA exists both as an evenly distributed organelle with basic functions, and as a highly differentiated subsynaptic organelle ensuring maturation and targeting of synaptic proteins. Finally, in the adult, denervation of a hemidiaphragm causes a burst of reexpression of all Golgi markers in extrasynaptic domains of the fibers, hence showing that the particular organization of the secretory pathway is placed under nerve control.

IN adult innervated muscle fibers, acetylcholine receptors (AChR)¹ are confined to the sarcolemmal membrane domain located under the nerve endings at a surface density of 10^4 molecules/ μm^2 (Salpeter and Loring, 1985). The mechanisms underlying the development and maintenance of this highly localized distribution of AChR are under intensive investigation (for reviews see Cartaud

and Changeux, 1993; Hall and Sanes, 1993; Duclert and Changeux, 1995). Typically, this organization becomes established during development in a sequence of steps that starts when motor nerves contact embryonic myotubes causing a clustering of AChRs in the myotube membrane (Andreson and Cohen, 1977; Role et al., 1985). The maintenance of such a high density of AChRs requires the renewal of AChR at synaptic sites (Fambrough, 1979), which may be achieved by local insertion of AChRs into the postsynaptic membrane (Role et al., 1985; Dubinsky et al., 1989; Stya and Axelrod, 1983). Moreover, the compartmentalization of AChR gene transcription at the level of subneural nuclei results in an accumulation of mRNAs encoding the various subunits of the AChR in the endplate region (Merlie and Sanes, 1985) at the level of the subjunctional “fundamental nuclei” (Fontaine and Changeux,

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; E, embryonic day of development determined from the day of fertilization; GA, Golgi apparatus; IC, intermediate compartment; MPR, M6PR, mannose-6-phosphate receptor; SSR, signal sequence receptor.

1989; Goldman and Staple, 1989; Brenner et al., 1990; Simon et al., 1992). A dual regulation of AchR expression, involving a nerve-mediated activation at the neuromuscular junction and extrajunctional repression by nerve-evoked electrical activity, contributes to this compartmentalization (reviewed in Changeux, 1991). Taken together, these data support the view that AchR proteins, as other synaptic proteins (Jasmin et al., 1993; Michel et al., 1994), may be synthesized and assembled in the synaptic region before being addressed to the postsynaptic membrane by a locally differentiated secretory machinery (for review see Cartaud and Changeux, 1993).

Until recently, however, little information existed about the secretory pathway in innervated muscle. Several studies have established that differentiated myogenic cells disclose specific features in their Golgi apparatus (GA) organization. Upon differentiation of myoblasts into myotubes, the GA undergoes a dramatic reorganization from a polarized juxtannuclear to a perinuclear location (Tassin et al., 1985; Miller et al., 1988). Such an atypical perinuclear organization was also observed in mononucleated cardiac myocytes (Kronebush and Singer, 1987), as well as in differentiated C2 cells grown in nonfusing conditions (Ralston, 1993). These latter experiments indicate that the reorganization of the Golgi complex results from the activation of the myogenic program, and occurs independently from myoblast fusion. The compartmentalization of the GA within the subsynaptic domain, which we observed in adult chick skeletal muscle, further points to a particular organization of the secretory pathway in innervated myofibers (Jasmin et al., 1989). Recently, using a set of anti-Golgi antibodies to the various subcompartments of the GA, we extended this notion to adult mammalian muscle. Indeed, markers corresponding to *cis*, *medial*, and *trans* cisternae of the GA systematically displayed a compartmentalized distribution within the adult fiber (Jasmin et al., 1995; but see Ralston, 1993). Moreover, the biochemical composition of the subsynaptic GA also differed from that commonly found in other cell types, several standard markers such as TGN38 and α -mannosidase II being undetected. Upon denervation, a relocation of the GA in association with many extrajunctional nuclei takes place 5 d after nerve section, consistent with the well-known process of denervation hypersensitivity (for reviews see Changeux, 1991; Hall and Sanes, 1993). Furthermore, subsets of cold-stable and acetylated microtubules are localized in the subneural domain of chick and rodent muscles (Jasmin et al., 1990; G. Camus, and J. Cartaud, unpublished data), a distribution consistent with the well-known role of microtubules in the organization of the secretory pathway (for review see Kreis, 1990).

Developmental studies in mouse diaphragm showed that mRNAs encoding α and γ subunits of the AchR become compartmentalized soon after neuromuscular contact was established in newly fused myofibers (Piette et al., 1993), while δ subunit compartmentalized expression is only effective at birth (Simon et al., 1992). Finally, expression of γ -subunit mRNA at the endplate level decreases, and is replaced by ϵ at the end of the first week after birth (Brenner et al., 1990). Since the developmental regulation of the AchR subunits in the membrane clearly parallels that of the mRNAs, one may hypothesize that in addition

to this transcriptional control by the nerve, a correlative developmental regulation also affects posttranscriptional steps involved in the biosynthesis, assembly, and targeting of synaptic components.

In this work, we focused on the remodeling of the secretory pathway in diaphragm muscle during early endplate formation. Using a set of antibodies mapping to the main compartments of membrane protein synthesis and transport from the RER to the plasma membrane, as well as to the endocytic pathway, we followed, by immunofluorescence microscopy, the reorganization of the entire secretory pathway in the fiber. Our results show that compartmentalization of the GA is achieved shortly after the onset of synaptogenesis, while RER, intermediate compartment (IC), and endocytic pathway remain unaffected by motor innervation. Denervation experiments point to the nerve-dependence of the process of compartmentalization of the GA. The data presented in this paper support a coordinated regulation of transcriptional and posttranscriptional events leading to the integrated organization of the subneural sarcoplasm under nerve control.

Materials and Methods

Tissue Preparation and Immunocytochemistry

C57BL6 mice were used. Embryonic day of development was determined from the day of fertilization as day 0 (E0), on the basis of morphological criteria (Rugh, 1990). Mice were killed by cervical dislocation, and the embryos or newborns were dissected in PBS for diaphragm collection. Fixation of the diaphragms was made by squeezing the samples between two glass slides with 4% paraformaldehyde for 20 min, then with 8% paraformaldehyde for a further 15 min after having removed one of the glass slides. From the flat muscle sheets thus obtained, small blocks (1.5–2 mm wide) were cut out to isolate either medial areas containing many synapses, or areas selected at the peripheral edge of the diaphragms. These samples were then infused in increasing sucrose solutions (from 0.5 to 2.1 M sucrose in PBS) before being mounted on aluminum supports and frozen in liquid nitrogen.

Semithin cryosections, 1–2.5 mm thick, were cut at -70°C using an ultracytome (FC4 with cryoattachment; Reichert Scientific Instruments, Buffalo, NY). To obtain an overall view of both the junctional and extrajunctional areas, the sections were cut in a parallel direction with respect to the plane of the diaphragm muscle. The cryosections were collected with drops of 2.1 M sucrose in a loop, and stuck on glass coverslips. After preincubation in PBS containing 1% BSA and 5% decomplexed goat serum, the sections were double-labeled for a chosen membranar organelle antigen and the AchR at the neuromuscular junction, followed by anti-rabbit secondary antibody (antiimmunoglobulin antibodies labeled with fluorescein; Cappel Laboratories, Malvern, PA) or rhodamine-labeled α -bungarotoxin, respectively (in some cases nuclei staining with 4'-6 diamidino-2-phenylindole dihydrochloride was also included). The coverslips finally were mounted in an antibleach/glycerol/PBS solution (Citifluor Ltd., London, UK).

Antibodies

Several antibodies directed against the various subcompartments of the GA were used in the present study. The medial-Golgi cisternae were labeled with either an affinity-purified rabbit antibody raised against a 160-kD medial-Golgi sialoglycoprotein (MG-160) (Gonatas et al., 1989; Croul et al., 1990), or with an affinity-purified rabbit antibody to α -mannosidase II (Moremen and Touster, 1985). The TGN was labeled using an affinity-purified rabbit antibody against TGN38, an integral membrane protein of the *trans*-Golgi network (Luzio et al., 1990). Affinity-purified rabbit anti-rab6p antibody was used to detect a ubiquitous small GTPase of the GA (Goud et al., 1990; Antony et al., 1992). Rabbit anti-signal sequence receptor (SSR) antibody was chosen to detect RER cisternae (Vogel et al., 1990), while a rabbit anticalcium-ATPase was used to specifically stain the sarcoplasmic reticulum (Enouf et al., 1988). Affinity-purified rabbit anti-rab1A p antibody, specific to the IC (Griffiths et al., 1994), was also used

to map the early secretory pathway. Late endosomal compartments were detected with an affinity-purified antibody against the cation-independent mannose 6-phosphate receptor (Griffiths et al., 1988).

Denervation

Denervation of diaphragm muscle was performed under diethyl-ether anesthesia of adult mice. A small incision was made between the ribs at the midthorax level. Using a glass hook passed between the ribs, the phrenic nerve was pulled and sectioned on one side only. The operated animals were killed after 5 d. The diaphragm muscle was dissected out, and cut in two hemidiaphragms providing denervated and innervated halves, the latter as a control.

Photography

Micrographs of the muscle sections were taken with an Aristoplan photomicroscope (E. Leitz, Inc., Rockleigh, NJ) equipped with epifluorescence illumination using Plan \times 63 (NA 1.40) and Plan \times 100 (NA 1.32) immersion optics. T-Max films (Eastman Kodak Co., Rochester, NY) were used and set at 800 ASA, and developed accordingly.

Results

Time Course of Compartmentalization of the Golgi Apparatus to the Subneural Domain

Until now, the organization of the GA in muscle fibers has been investigated in adult tissues (Jasmin et al., 1989, 1995). To investigate the time course of the GA compartmentalization, we collected diaphragms from 14.5-d embryos (E14.5), shortly after the onset of innervation (at E12–E13), up to E19. Semithin cryosections performed in the plane of the muscle sheet (see Materials and Methods) were submitted to immunocytochemical labeling with anti-Golgi antibodies and RITC-coupled α -bungarotoxin.

In diaphragm sections from E14 embryos labeled with an antibody to the medial-Golgi cisternae (MG-160, see Materials and Methods), numerous spots could be seen scattered along the fibers, in both junctional and nonjunctional areas (Fig. 1 *A*). Yet, more obvious was the distribution pattern observed at E16, as diaphragms thicken and larger synapse stripes become apparent. Again, the distribution of the Golgi antigens corresponded to spots spread all over the plane of the sectioned fibers (Fig. 1 *B*). From E17.5, the immunoreactivity had disappeared from the extrajunctional areas and codistributed with neuromuscular junction labeled with α -bungarotoxin (Fig. 1 *C*). This pattern resembled the compartmentalized adult pattern in rat limb muscles (Jasmin et al., 1995). Similar compartmentalization of the Golgi complex in the subsynaptic area was also observed using an antibody directed against the small GTPase rab6p, a ubiquitous marker of the Golgi complex (Fig. 2 *A*). Therefore, the compartmentalization of the GA in muscle fibers follows by 2–3 d the onset of synaptogenesis and the compartmentalization of AchR mRNAs (Piette et al., 1993).

Biochemical Differentiation of the Subs synaptic GA during Development

In mouse and rat muscle cell lines, C2 and L6, the GA was positively labeled with all tested, bona fide anti-Golgi antibodies including TGN38 and α -mannosidase II (Jasmin et al., 1989, 1995; Ralston, 1993). Surprisingly, in mature rat muscle, no immunoreactivity was detected with α -man-

nosidase II (Ralston, 1993) or with TGN 38 (Jasmin et al., 1995), even in the subneural domain. In the present study, at E19, a developmental stage at which a differentiated subneural GA was achieved (Fig. 2 *A*), we noticed the absence of immunoreactivity for both TGN38 and α -mannosidase II markers, especially in the subneural area (Fig. 2, *B* and *C*). Thus, this biochemical differentiation of the GA occurs quickly after the onset of synaptogenesis, like the compartmentalization shown for other GA markers (see above).

A striking feature of the GA relocation during muscle development and synaptogenesis is the “fading” of the GA from extrajunctional areas. The dynamics of this phenomenon were observed in the peripheral area of E19 developing diaphragms, where extensive incorporation of myoblasts occurs by fusion at the ends of the growing fibers (Fig. 3, *A* and *B*). In this myoblast-rich area of the diaphragm, we detected typical juxtannuclear labeling using anti-rab6p antibodies (Fig. 3 *A*, *arrows*). Interestingly, from the periphery toward the medial zone of the developing diaphragm, rab6p labeling disclosed a gradient of GA pattern turning from a spotty myoblast-like pattern at the periphery (Fig. 3 *A*) to a faint and dispersed dotted pattern in the more central region of the fibers. These observations evoke the involvement of a factor causing the repression and/or dispersion of the GA in the developing myofiber (see Discussion).

Early Secretory Pathway Escapes Compartmentalization in the Muscle Fiber

To test whether the early compartments involved in the secretory pathway also redistribute upon innervation, we investigated both the distribution of the RER, i.e., the site of membrane protein synthesis, and that of the IC, a transition element which directs proteins leaving the RER to the GA, and which is involved in the retrieval of RER components (for review see Hauri and Schweitzer, 1992).

Anti-SSR antibody (Vogel et al., 1990) was chosen to assess the overall distribution of the RER cisternae. In diaphragm muscle collected from 8-d-old mice, a strong labeling was displayed, mostly in a perinuclear disposition along the entire fiber, both in junctional and extrajunctional domains (Fig. 4 *B*). To rule out any possible confusion between the RER and the abundant sarcoplasmic reticulum of the myofibers, we compared the SSR pattern of staining with that obtained with anti- Ca^{2+} ATPase antibody, a marker for the sarcoplasmic reticulum. This last antibody gave a typical grid-shaped pattern of staining in myofibers. The last two patterns of staining were mutually exclusive, and reflected the physical separation of the two biochemically and functionally specialized ER domains in muscle fibers (Fig. 4, *B* and *D*).

Given the striking disparity between the location of early compartments of the biosynthetic pathway and the GA, it was of interest to investigate the distribution of the IC. As expected from the known distribution of the IC in most cells, anti-rab1A p antibody, which labels mostly this compartment (Griffiths et al., 1994), gave an even spotty pattern of labeling distributed all over the fibers (Fig. 5). No restrictive concentration of rab1A p-immunoreactivity to the subneural sarcoplasm could be found.

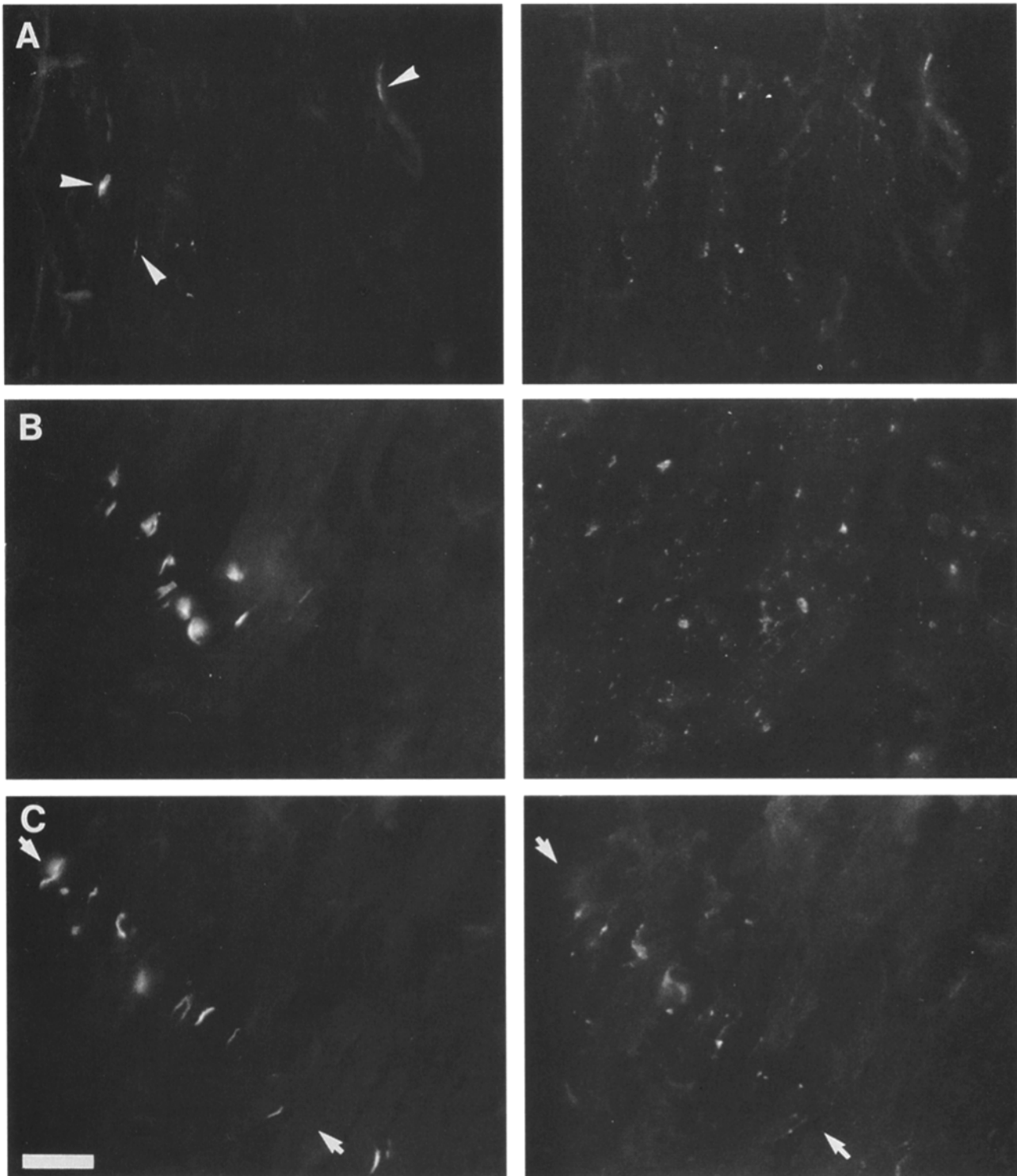


Figure 1. Time course of compartmentalization of the GA in the subneural domain of developing diaphragm. Cryosections were made in the plane of embryonic diaphragms taken at various developmental stages and were double-labeled with rhodamine-conjugated α -bungarotoxin (*synapse localization, left panels*) and anti-medial Golgi antibody (*MG-160, right panels*). (A) At E14, few synapses and numerous Golgi spots were observed scattered over the whole section. (B) At E16, although larger and more numerous synapses were detected, the Golgi pattern of staining was distributed over the whole section as in earlier stages. (C) At E17, characteristic coalignment of Golgi immunoreactivity with the synapses location was observed (*arrows*), thus showing that at this time of embryonic development, the GA was already compartmentalized in the subsynaptic domain. Bar, 20 μ m.

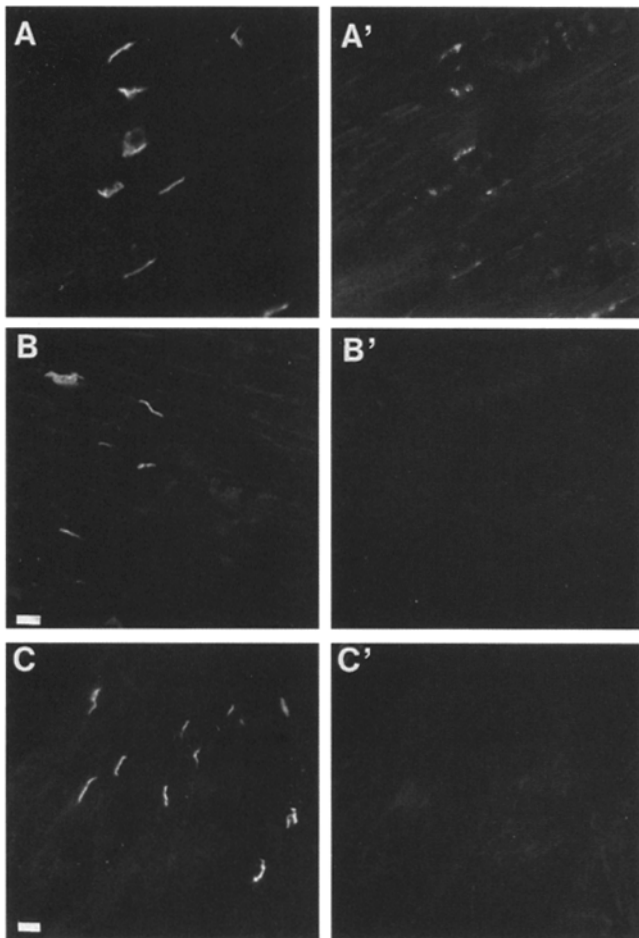


Figure 2. The compartmentalization of the GA is associated with a biochemical differentiation. (A, A') E19 diaphragm sections were double-labeled with α -bungarotoxin (A) and anti-rab6p antibody (A'). The Golgi complexes accumulated in the subneural sarcoplasm (A') under the synapses location (A). In contrast, no immunoreactivity was detected with antibodies directed against anti-TGN38 (B'), or against α -mannosidase II (C'), in the synaptic, as well as extrasynaptic areas (B and C), hence indicating a biochemical differentiation of the subneural GA. Bars, 20 μ m.

Mannose-6 Phosphate Receptor-rich Compartment (MPR or Prelysosomal Compartment) Does not Display Compartmentalization in the Innervated Fiber

To investigate further the distribution of other crucial compartments of membrane traffic pathways in the myofibers, we selected a well-known marker of the endocytic pathway, the MPR. This receptor mediates the transport and delivery of lysosomal enzymes from the GA to the endocytic pathway (von Figura and Hasilik, 1986). The bulk of the receptor was localized to a prelysosomal compartment that serves as an intermediate compartment where lysosomal enzymes are released from the MPR, the so-called MPR-rich compartment, or prelysosomal compartment (Griffiths et al., 1988). In E19 diaphragms, using the anti-MPR antibody, we found the immunoreactivity evenly scattered, with both a perinuclear and spotty staining along the fibers (Fig. 6), thus indicating that the prelysosomal compartment exhibits no compartmentalization in the diaphragm fibers.

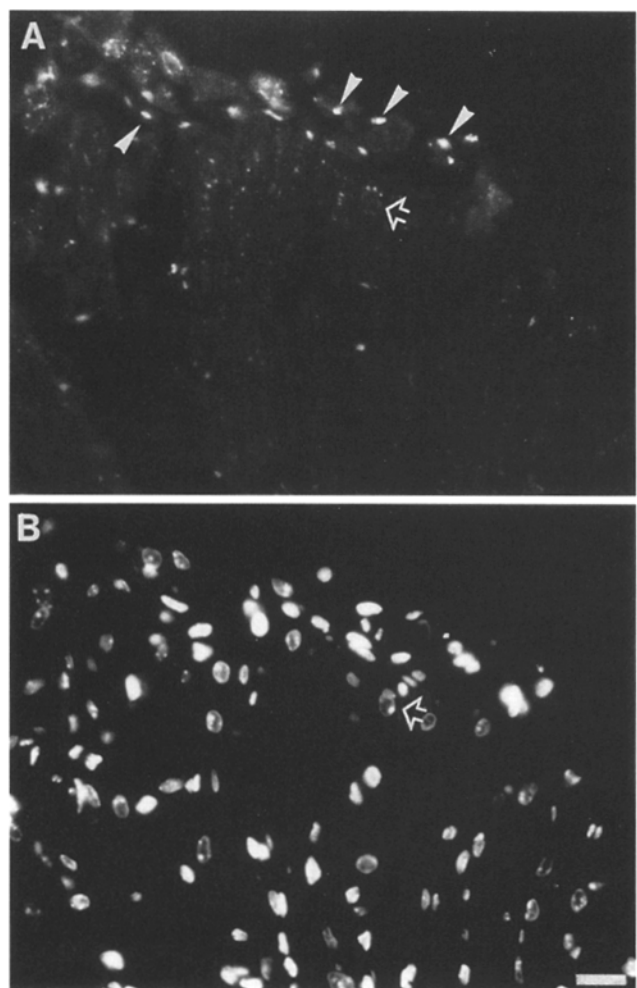


Figure 3. Edge of a diaphragm at E19 labeled with anti-rab6p antibody. (A) The top part of the figure corresponds to a peripheral zone of the diaphragm muscle where extensive fusion of myoblasts with the developing myofibers occurs. The juxtannuclear Golgi pattern of staining in myoblasts (arrows) contrasted with the faint spotty pattern at the ends of the fibers which progressively faded away from the diaphragm's edge. Note the perinuclear GA pattern (open arrow) reminiscent of the GA organization prevailing in myotubes. (B) Nuclear staining with 4'-6-diamidino-2-phenylindole dihydrochloride of the same field. Bar, 20 μ m.

In Vivo Denervation Elicits a Burst of Reexpression of GA Markers

To assess the role played by the motor nerve in the reorganization of the membrane traffic in differentiated muscle fibers compared with myotubes, we investigated the changes occurring after denervation of the diaphragm in adult mouse. Denervation of the diaphragm can be performed on a hemidiaphragm, leaving intact the opposite half as a contralateral control in the same animal. 5 d after denervation, the immunoreactivity was tested on semithin cryosections of both denervated and control hemidiaphragms using the same panel of anti-Golgi antibodies as previously. Immunolabeling of the sections with any of the GA antibodies resulted in a striking burst of immunoreactivity (Fig. 7, left panels) compared with the control hemidia-

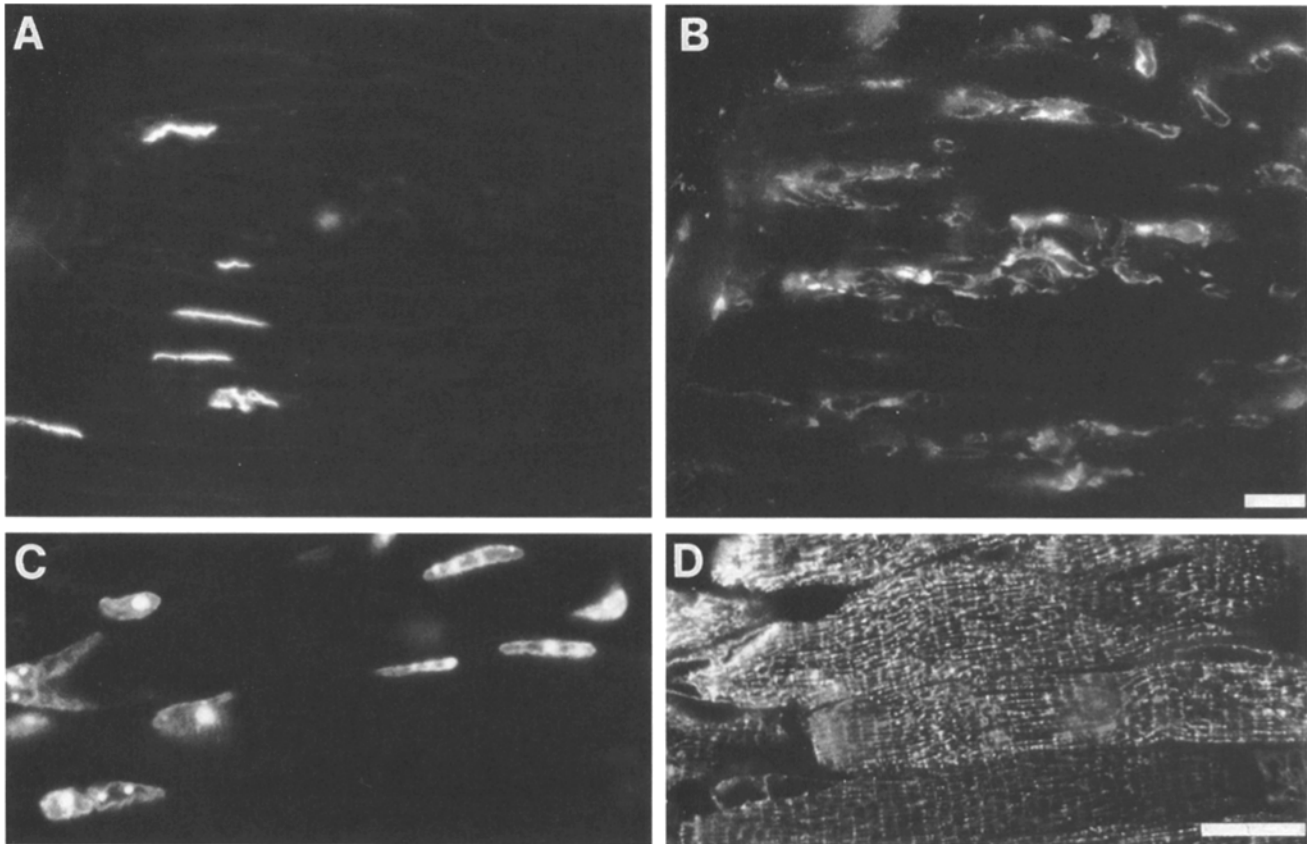


Figure 4. Distribution of the RER in innervated mouse-diaphragm fibers (8-d-old) using an anti-SSR antibody. A perinuclear pattern of staining was disclosed in the fibers (anti-SSR staining in *B*) independently of the synapses' location (α -bungarotoxin labeling in *A*), and therefore did not appear compartmentalized. The sarcoplasmic reticulum labeled with anticalcium ATPase antibody (*D*) gave quite a distinct pattern. (*C*) nuclear staining with 4'-6 diamidino-2-phenylindole dihydrochloride of the same field. Bars, 20 μ m.

phragm (Fig. 7, *right panels*). Particularly striking were the immunofluorescence patterns obtained with anti-rab6p, anti-MG-160, and anti-TGN 38 antibodies, which disclosed a punctate perinuclear staining in a large fraction of the fibers. These patterns strikingly resembled those observed in myotubes. Mannosidase II gave a different pattern of reexpression, less punctate, with larger spots. In addition, reexpression also affected a population of cells which displayed myoblast-like Golgi patterns and included most probably satellite cells.

Discussion

The recent observations by Jasmin et al. (1995) reported striking differences in the distribution and biochemical composition of the GA in adult rodent myofibers compared with cultured myogenic cells. This organelle, which is involved in the transport and targeting of membrane proteins, displays a compartmentalized organization in innervated muscle fibers, thus suggesting a possible role for the posttranslational processing of synaptic proteins. Changes in the spatial distribution of AchR along muscle fibers during the first steps of synaptogenesis are accompanied by profound remodeling in the pattern of expression of several genes, in particular, those encoding AchR subunits and acetylcholine esterase in junctional and extrajunctional regions of the muscle fibers. Therefore, we assume

that a coordinated regulation at both transcriptional and posttranslational levels is involved in the genesis and maintenance of the postsynaptic membrane differentiation (Cartaud and Changeux, 1993). In mouse diaphragm, a compartmentalized expression of the α and γ subunit mRNAs occurs as early as E13.5, when the first neuromuscular contacts are formed (Piette et al., 1993). In this work we focused on an important posttranslational processing of membrane proteins, i.e., the secretory pathway, and followed the developmental aspects of the reorganization of membrane traffic along the biosynthetic pathway with respect to motor innervation.

GA Subcompartments Relocalize Soon after Motor Innervation during Development of Mouse Diaphragm

In the course of early development of the diaphragm muscle, soon after innervation, most of the selected GA markers mapping to various cisternae of the GA accumulate in the subneural sarcoplasm, while immunoreactivity in the extrasynaptic areas progressively fades. This compartmentalization, as followed by the medial-Golgi marker MG-160, is chronologically close to that described for the spatial expression of the mRNAs encoding the AchR subunits, although with a delay of $\sim 2-3$ d.

Another interesting observation was made from examining the edges of embryonic diaphragm where fusion of

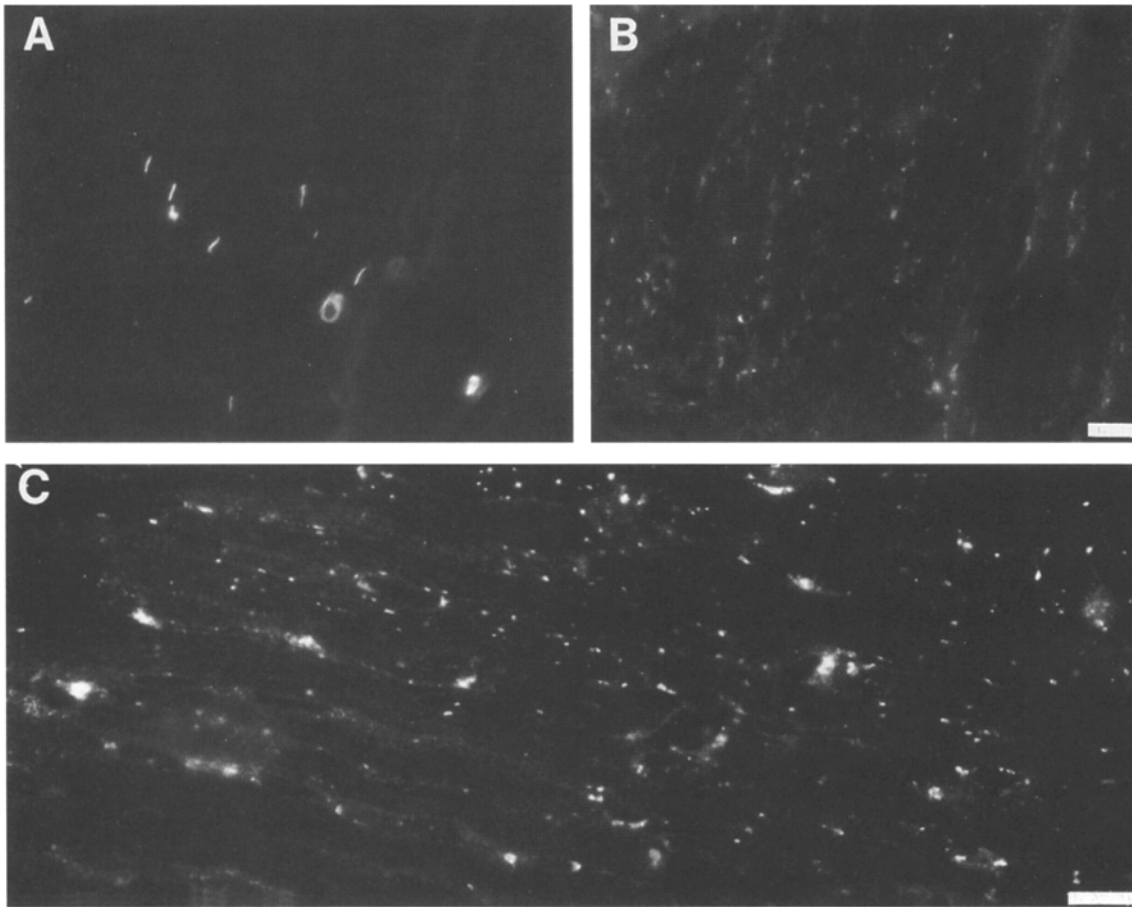


Figure 5. Labeling of the IC with anti-rab1A p antibody in E19 embryonic diaphragm. A spotty pattern of staining was evenly distributed over the sections (*B*) irrespectively of the subneural and extrajunctional domains (α -bungarotoxin staining in *A*). (*C*) Detailed view of rab1Ap pattern of staining in an extrajunctional field. Therefore, similarly to the RER, the distribution of the IC was not restricted to the subneural domain. Bars, 20 μ m.

myoblasts into developing myofibers takes place. Evidence for a graded fading of the immunoreactivity was well-illustrated by the pattern of rab6p staining observed in this particular area. The contrast between the myoblast-like pattern and the weak, spotty pattern at the ends of the fibers suggests that the innervated fiber carries a signaling factor responsible for the relocation of the GA. Such components were already postulated from heterocaryons studies. Actually, human hepatocytes fused with mouse muscle cells trigger major changes in cytoarchitecture, particularly in the organization of the GA of the hepatocyte cells that turn to a muscle cell configuration, thereby showing that in the heterokaryon muscle cell components dictate the expression of a muscle cell-type program (Miller et al., 1988). It thus seems that mature sarcoplasm is endowed with special properties with respect to Golgi-apparatus organization. In this cellular context, a consequence of motor innervation would be (other than its role in regulating gene expression within subneural nuclei) to locally reorganize the distal part of the biosynthetic pathway. The observation of a particular microtubular network, composed of cold-resistant and acetylated microtubules located in the subneural sarcoplasm (Jasmin et al., 1990), provides a clue for a synapse-related organization of the subneural exocytic pathway.

Concomitant to the compartmentalization of the GA is a biochemical differentiation of this organelle, already mentioned in the case of adult rat muscle (Jasmin et al., 1995; Ralston, 1993). The disappearance of TGN 38 and α -mannosidase II immunoreactivities is already achieved at the earliest days of synaptogenesis (as soon as E14–E16). This shows that both the “repressed expression” of these latter GA markers, and the compartmentalization of other markers occur concomitantly during myogenesis. As reported by Moremen (Moremen and Robbins, 1991), detection of α -mannosidase II mRNAs failed in skeletal muscle tissue, whereas it was positive in all other examined tissues in rat. This indicates that in our experiments, the lack of detection of this enzyme in skeletal muscle most likely results from a regulation at the transcriptional level. Interestingly, in *Torpedo californica*, the state of glycosylation of the AchR subunits is of high mannose type, (Man)8 (GlcNAc)2 and (Man)9 (GlcNAc)2, which indicates that the oligosaccharide substrate was at least partially exposed to α -mannosidase II enzyme (Nomoto et al., 1986; Strecker et al., 1994). These data suggest that mature muscle cells possess a muscle-specific isoform of α -mannosidase II that is not detected by any current probes.

The notion of a biochemical differentiation within the subneural GA was already emphasized by synapse-specific

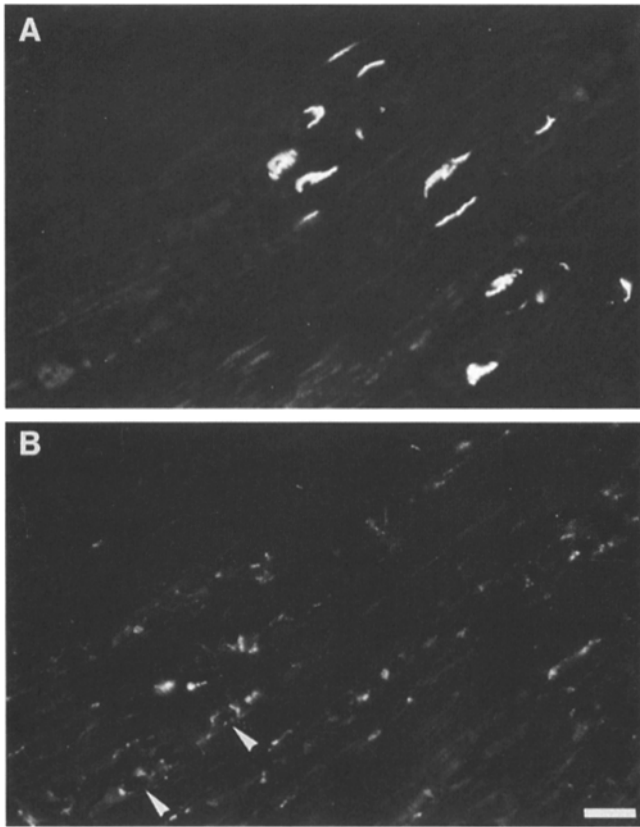


Figure 6. Distribution of the mannose 6-phosphate receptor-rich compartment (late endosome). The pattern of labeling obtained with the anti-M6PR antibody (*B*) was observed over the whole fiber, often in a perinuclear disposition (*arrows, B*). No codistribution with synaptic areas (α -bungarotoxin labeling, *A*) was observed. Bar, 20 μ m.

glycosylations (Scott et al., 1988; Iglesias et al., 1992) as well as synapse-specific localization of *N*-acetylgalactosaminyl transferase in skeletal muscle (Scott et al., 1989).

Early Exocytic and Endocytic Compartments Escape Restricted Subneural Redistribution during Diaphragm Development

To understand further the organization of the exocytic and endocytic pathways in adult and developing diaphragm muscle, we have extended our analysis to the distribution of selected markers of various membrane subcompartments of these pathways. We focused on the early compartments of the secretory pathway, i.e., the RER and the IC. Using anti-SSR antibody to specifically detect the RER, we showed that the pattern of staining, which was mostly perinuclear, did not display compartmentalization in embryos examined at postnatal day 8 (2 wk after the compartmentalization of the GA occurred). So was the case for the IC detected with anti-rab1Ap antibodies (Griffiths et al., 1994). In E19 embryonic diaphragm, we showed that this compartment distributes evenly along the fibers with a spotty pattern. Most interestingly, these observations on the early compartments of the secretory pathway bring evidence for a spatial codistribution of the RER and IC in contrast with the segregation of most of

the GA in the subneural region. Therefore, because of the compartmentalization of the GA only, this model provides a clue for a physical discontinuity between the IC and the entry into the GA (*cis*-Golgi). These data support the hypothesis, among others (for review see Hauri and Schweizer, 1992), that a physical link exists between the RER and the IC, the latter being a possible extension of the former (Griffiths et al., 1994).

Our present data also bring evidence that endocytic pathway (late endosome) labeled with anti-MPR antibody is evenly distributed over the entire muscle fiber. The MPR is involved in the sorting of lysosomal enzymes from the secretory pathway at the TGN level (von Figura and Hasilik, 1986; Griffiths et al., 1989) to be addressed to the endocytic compartments. The noncompartmentalized distribution of the MP6R-rich compartment suggests that this "housekeeping" role is not functionally related to the specialized subneural GA, but rather related to the scattered extrasynaptic GA. Therefore, we propose that in the innervated myofibers the GA may exist both (*a*) as an evenly distributed organelle assuming basic functions such as secretion of muscle glycoproteins, and (*b*) as a subneural highly differentiated GA specialized in the maturation and targeting of synaptic proteins.

Spatial Pattern of Distribution of Golgi Markers Is Dependent on Motor Innervation

In this work, we showed that denervation of a hemidiaphragm in adult mouse reverses both the distribution of some GA markers and the repression of TGN38 and α -mannosidase II. All GA markers whose distribution was restricted to the subneural domain in adult innervated muscle reappeared, significantly, in extrajunctional regions of the fibers upon denervation, together with TGN38 or α -mannosidase II, which were extinguished in both subsynaptic and extrasynaptic regions. Thus, in denervated muscle fibers, the GA recovers the pattern prevailing at early steps of myogenic differentiation. From these observations, we conclude that the spatial organization and biochemical specialization of the secretory pathway in mature myofibers is placed under motor-nerve control.

Denervation of innervated muscle is known to induce drastic changes in the distribution and properties of synaptic molecules, reversing the sequence of events during development. Concerning the AchR, the dual regulation of gene expression in junctional versus extrajunctional regions of the myofibers is readily abolished upon denervation, leading to a myotube-like pattern of gene expression (for reviews see Salpeter and Loring, 1985; Changeux, 1991). Our data are consistent with this notion. One may thus hypothesize that the regulation of the expression of GA components may result from a nerve-dependent transcriptional control similar, if not identical, to that described for AchR.

Concluding Remarks: Functional Implication of Membrane Reorganization for Synaptogenesis

The maintenance of a high concentration of AchRs at the synapse requires replacement of AchRs at synaptic sites. There is evidence that this is accomplished, at least partly, by local insertion of newly synthesized AchRs into the

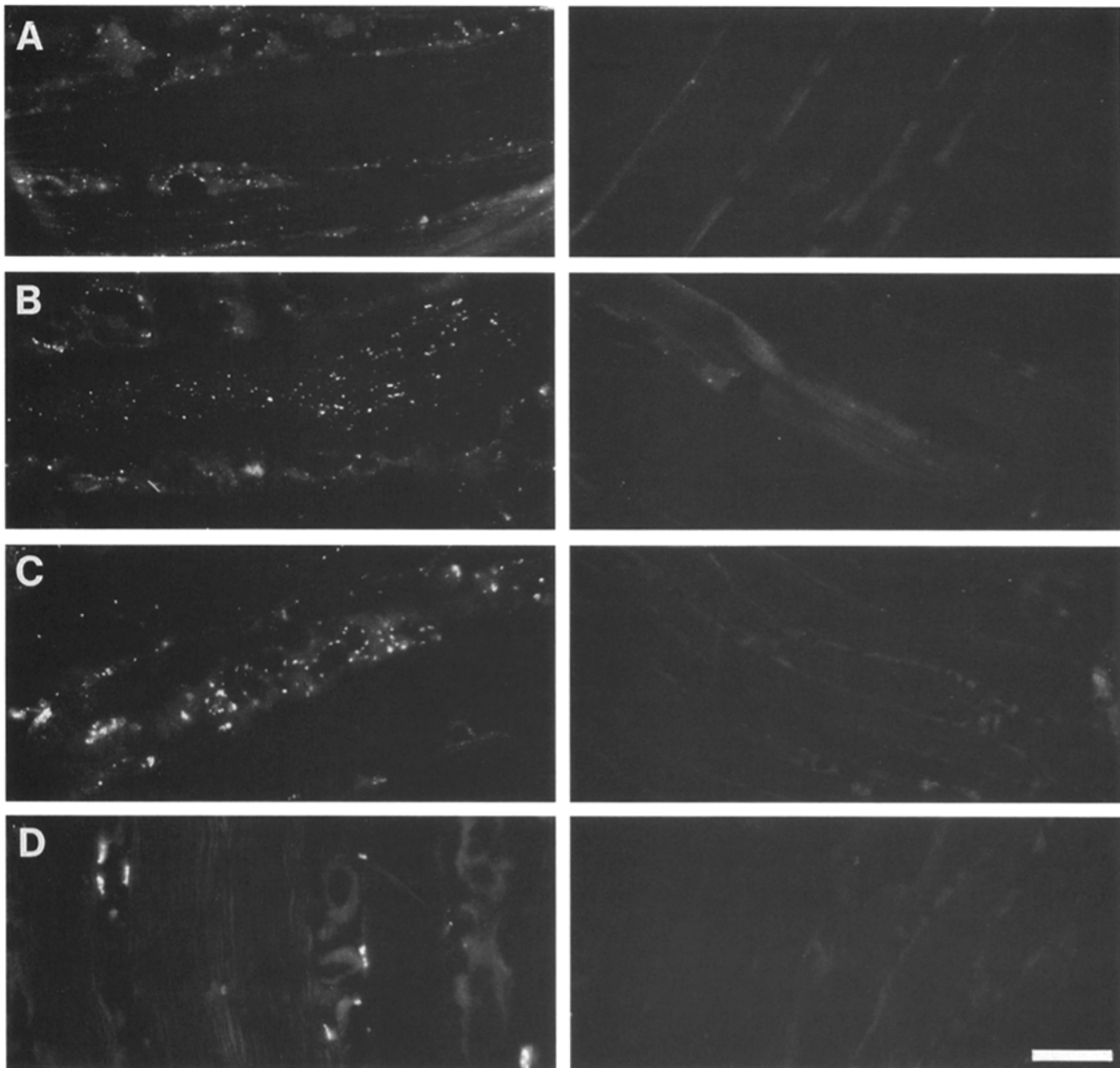


Figure 7. Reexpression of GA markers in 5-d denervated hemidiaphragm. 5 d after denervation, sections were made in extrasynaptic areas, both in the denervated (*left panels*) and contralateral innervated (*right panels*) hemidiaphragms, and stained with various antibodies to the Golgi complex. A burst of reexpression was observed in the denervated hemidiaphragm with all Golgi markers. Note the characteristic myotube-like perinuclear pattern of staining of the GA (particularly obvious in *A*, *B*, and *C*). The contralateral innervated hemidiaphragm did not display any significant immunoreactivity in the extrajunctional areas. (*A*) rab6p; (*B*) MG-160; (*C*) TGN38; (*D*) α -mannosidase II. Bar, 20 μ m.

postsynaptic membrane (Role et al., 1985). Since mRNAs encoding the different subunits are highly concentrated at synaptic sites in adult myofibers, AchR polypeptides are likely to be synthesized locally in the synaptic region. Our observations represent the first demonstration that a specialized machinery for synthesis, assembly, sorting, and targeting of membrane glycoproteins indeed exists in the subneural sarcoplasm, and is under the control of the motor nerve. The existence of a pool of discrete Golgi membranes (Andersson-Cedergren, 1959; Ralston, 1993) distributing all over the myofiber may provide the cell with an undifferentiated GA, assuming the transport of ubiqui-

tous membrane proteins contrasting with the specialized subneural GA.

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References

- Andersson-Cedergrén, E. 1959. Ultrastructure of motor endplate and sarcolemmal components of mouse skeletal muscle fibers as revealed by three-dimensional reconstructions from serial sections. *J. Ultrastruct. Res. Suppl.* 1: 1-19.
- Antony, C., C. Cibert, G. Géraud, A. Santa Maria, B. Maro, V. Mayau, and B. Goud. 1992. The small GTP-binding protein rab6p is distributed from medial Golgi to the trans-Golgi network as determined by confocal microscopy approach. *J. Cell Sci.* 103:785-796.
- Andreson, M. J., and M. W. Cohen. 1977. Nerve induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.)* 268:757-773.
- Brenner, H. R., V. Witzemann, and B. Sakmann. 1990. Imprinting of acetylcholine receptor message RNA accumulation in mammalian neuromuscular synapse. *Nature (Lond.)* 344:544-547.
- Cartaud, J., and J. P. Changeux. 1993. Post-transcriptional compartmentalization of acetylcholine receptor biosynthesis in the subneural domain of muscle and electrocyte junctions. *Eur. J. Neurosci.* 5:191-202.
- Changeux, J.-P. 1991. Compartmentalized transcription of acetylcholine receptor genes during motor endplate epigenesis. *New Biol.* 3:413-429.
- Croul, S., S. G. E. Meztis, A. Stieber, Y. Chen, J. O. Gonatas, B. Goud, and N. K. Gonatas. 1990. Immunocytochemical visualization of the Golgi apparatus in several species, including human, and tissues with an antiserum against MG-160, a sialoglycoprotein of rat Golgi apparatus. *J. Histochem. Cytochem.* 38: 957-963.
- Dubinsky, J. M., D. J. Loftus, G. D. Fischbach, and E. L. Elson. 1989. Formation of acetylcholine receptor clusters in chick myotubes: migration or new insertion? *J. Cell Biol.* 109:1733-1743.
- Duclert, A., and J.-P. Changeux. 1995. Acetylcholine gene expression at the developing neuromuscular junction. *Physiol. Rev.* 75:339-368.
- Enouf, J., A.-M. Lompré, R. Bredoux, N. Bourdeau, D. de La Bastie, and S. Levy-Toledano. 1988. Different sensitivity to trypsin of the human platelet plasma and intracellular membrane Ca^{2+} pumps. *J. Biol. Chem.* 263:13922-13929.
- Fontaine, B., and J.-P. Changeux. 1989. Localization of nicotinic acetylcholine receptor α -subunit transcripts during myogenesis and motor endplate development in the chick. *J. Cell Biol.* 108:1025-1037.
- Fambrough, D. 1979. Control of acetylcholine receptors in skeletal muscle. *Physiol. Rev.* 59:165-227.
- Goldman, D. J., and J. Staple. 1989. Spatial and temporal expression of acetylcholine receptor RNAs in innervated and denervated rat soleus muscle. *Neuron* 7:649-658.
- Gonatas, J. O., S. G. Meztis, A. Stieber, B. Fleischer, and N. K. Gonatas. 1989. MG-160, a novel sialoglycoprotein of the medial cisternae of the Golgi apparatus. *J. Biol. Chem.* 264:646-653.
- Goud, B., A. Zahraoui, A. Tavistean, and J. Saraste. 1990. Small GTP-binding protein associated with Golgi cisternae. *Nature (Lond.)* 345:553-556.
- Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose 6-phosphate and the biogenesis of lysosomes. *Cell* 52:329-341.
- Griffiths, G., M. Ericsson, J. Krijnse-Locker, T. Nilsson, B. Goud, H.-D. Sölnig, B. L. Tang, S. H. Wong, and W. Hong. 1994. Localization of the Lys, Asp, Glu, Leu tetrapeptide receptor to the Golgi complex and the intermediate compartment in mammalian cells. *J. Cell Biol.* 127:1557-1574.
- Hall, Z. W., and J. R. Sanes. 1993. Synaptic structure and development: the neuromuscular junction. *Cell* 72:99-121.
- Hauri, H.-P., and A. Schweizer. 1992. The endoplasmic reticulum-Golgi intermediate compartment. *Curr. Opin. Cell Biol.* 4:600-608.
- Iglesias, M., J. Ribera, and J. E. Resquesda. 1992. Treatment with digestive agents reveals several glycoconjugates specifically associated with rat neuromuscular junction. *Histochemistry* 97:125-131.
- Jasmin, B. J., J. Cartaud, M. Bornens, and J.-P. Changeux. 1989. Golgi apparatus in chick skeletal muscle: changes in its distribution during end plate development and after denervation. *Proc. Natl. Acad. Sci. USA* 86:7218-7222.
- Jasmin, B. J., J. P. Changeux, and J. Cartaud. 1990. Compartmentalization of cold-stable and acetylated microtubules in the subsynaptic domain of chick skeletal muscle fibre. *Nature (Lond.)* 344:673-675.
- Jasmin, B. J., R. K. Lee, and R. L. Rotundo. 1993. Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. *Neuron* 11:467-477.
- Jasmin, B. J., C. Antony, J.-P. Changeux, and J. Cartaud. 1995. Nerve dependent plasticity of the Golgi complex in skeletal muscle fibers: compartmentalization within the subneural sarcoplasm. *Eur. J. Neurosci.* 7:470-479.
- Kreis, T. E. 1990. Role of microtubules in the organization of the Golgi apparatus. *Cell Motil. Cytoskeleton* 15:67-70.
- Kronebusch, P. J., and S. J. Singer. 1987. The microtubule-organizing complex and the Golgi apparatus are co-localized around the entire nuclear envelope in interphase cardiac myocytes. *J. Cell Sci.* 88:25-34.
- Luzio, J. P., B. Brake, G. Banting, K. E. Howell, P. Braghetta, and K. K. Stanley. 1990. Identification, sequencing and expression of an integral membrane protein of the trans-Golgi network (TGN 38). *Biochem. J.* 270:97-102.
- Merlie, J. P., and J. R. Sanes. 1985. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibres. *Nature (Lond.)* 317:66-68.
- Michel, R. N., C. Q. Vu, W. Tetzlaff, and B. J. Jasmin. 1994. Neural regulation of acetylcholinesterase mRNAs at mammalian neuromuscular synapses. *J. Cell Biol.* 127:1061-1069.
- Miller, S. C., G. K. Pavlath, B. T. Blakely, and H. Blau. 1988. Muscle cell components dictate hepatocyte gene expression and the distribution of the Golgi apparatus in heterokaryons. *Genes & Dev.* 2:330-340.
- Moremen, K. W., and P. W. Robbins. 1991. Isolation, characterization, and expression of cDNAs encoding murine α -mannosidase II, a Golgi enzyme that controls conversion of high mannose to complex N-glycans. *J. Cell Biol.* 115: 1521-1534.
- Moremen, K. W., and O. Touster. 1985. Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 cells. *J. Biol. Chem.* 260:6654-6662.
- Nomoto, H., N. Takahashi, Y. Nagaki, S. Endo, Y. Arata, and K. Hayashi. 1986. Carbohydrate structure of acetylcholine receptor from *Torpedo californica* and distribution of oligosaccharides among the subunits. *Eur. J. Biochem.* 157:233-242.
- Piette, J., M. Huchet, A. Duclert, A. Fujisawa-Sehara, and J.-P. Changeux. 1993. Localization of mRNAs coding for CMD1, myogenin and the α -subunit of the acetylcholine receptor during skeletal muscle development in the chicken. *Mech. Dev.* 37:95-106.
- Ralston, E. 1993. Changes in architecture of the Golgi complex and other subcellular organelles during myogenesis. *J. Cell Biol.* 120:399-409.
- Role, L. W., V. R. Matossian, R. J. O'Brien, and G. D. Fishbach. 1985. On the mechanism of acetylcholine receptor accumulation at newly formed synapses on chick myotubes. *J. Neurosci.* 5:2197-2204.
- Rugh, R. 1990. *The Mouse. Its Reproduction and Development*. Oxford University Press, Oxford, UK. 324 pp.
- Salpeter, M., and R. H. Loring. 1985. Nicotinic acetylcholine receptors in vertebrate muscle: properties, distribution and neural control. *Prog. Neurobiol. (NY)* 25:297-325.
- Scott, L. J. C., F. Bacou, and J. R. Sanes. 1988. A synapse-specific carbohydrate at the neuromuscular junction: association with both acetylcholinesterase and a glycolipid. *J. Neurosci.* 8:932-944.
- Scott, L. J. C., J. Balsamo, J. R. Sanes, and J. Lilien. 1990. Synaptic localization and neural regulation of an N-acetylglucosaminyl transferase in skeletal muscle. *J. Neurosci.* 10:346-350.
- Simon, A. M., P. Hoppe, and S. J. Burden. 1992. Spatial restriction of AChR gene expression to subsynaptic nuclei. *Development (Camb.)* 114:545-553.
- Strecker, A., P. Francke, C. Weise, and F. Hucho. 1994. All potential glycosylation sites of the nicotinic acetylcholine receptor δ subunit from *Torpedo californica* are utilized. *Eur. J. Biochem.* 220:1005-1011.
- Stya, M., and D. Axelrod. 1983. Diffusely distributed acetylcholine receptors can participate in cluster formation on cultured rat myotubes. *Proc. Natl. Acad. Sci. USA* 80:449-453.
- Tassin, A. M., M. Paintrand, E. G. Berger, and M. Bornens. 1985. The Golgi apparatus remains associated with microtubule organizing centers during myogenesis. *J. Cell Biol.* 101:630-638.
- von Figura, K., and A. Hasilik. 1986. Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* 55:167-193.
- Vogel, F., E. Hartmann, D. Görlich, and T. Rapoport. 1990. Segregation of the signal sequence receptor protein in the rough endoplasmic reticulum membrane. *Eur. J. Cell Biol.* 53:197-202.